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Characterization of physical entrapment and chemical conjugation of adriamycin in polymeric micelles and their design for in vivo delivery to a solid tumor

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Abstract

An anticancer drug adriamycin (ADR) was incorporated into polymeric micelles forming from poly(ethylene glycol)-poly(aspartic acid) block copolymer by chemical conjugation and physical entrapment. Structural stability of the polymeric micelles was found to be dependent on both the contents of chemically conjugated and physically entrapped ADR. The polymeric micelle with high contents of the chemically conjugated ADR and the physically entrapped ADR expressed very high in vivo antitumor activity against murine C 26 tumor, while the polymeric micelle with only the chemically conjugated ADR showed negligible in vivo activity. This indicates that the physically entrapped ADR played a major role in antitumor activity in vivo. For the polymeric micelle with the high ADR contents, it was found that a dimer of adriamycin molecules formed and that this dimer was physically entrapped in the inner core of the micelle as well as intact ADR. © 1998 Elsevier Science B.V.

Keywords: Polymeric micelles; Stability; Anticancer drug; Adriamycin; Dimer

*Corresponding authors.

Abbreviations: ADR, adriamycin; ADR·HCl, adriamycin hydrochloride; DAU, daunomycin; N-Ac ADR, N-acetyl adriamycin; N-Ac DAU, N-acetyl daunomycin; EDC·HCl, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride; Asp, aspartic acid; PEG, poly(ethylene glycol); P(Asp), poly(aspartic acid); PEG-P(Asp), poly(ethylene glycol)-poly(aspartic acid) block copolymer; PEG-P(Asp(ADR)), adriamycin-conjugated poly(ethylene glycol)-poly(aspartic acid) block copolymer; HPLC, high-performance Liquid phase Chromatography; GPC, gel-permeation chromatography; DMF, N,N-dimethylformamide

1. Introduction

Drug delivery systems with long-circulating characteristics [1] in the bloodstream have caught much attention not only for chemo-therapeutic purposes [2] but also for diagnostic imaging. Delivery systems based on liposomes [3,4], microspheres [5], and polymeric micelles [6–9] have been studied for these purposes. Although polymeric micelle systems

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have only several years' history of research for in vivo applications [10,11], significant enhancement of in vivo activity of an anticancer drug was obtained [6,12]. As compared with other types of carrier systems, the polymeric micelle systems possess several benefits [13,14] including the following important two characteristics. The first characteristics is wide applicability of the polymeric micelle systems to drugs, since drugs can be incorporated into the micelles both by chemical conjugation and physical entrapment. Particularly, physical entrapment utilizing hydrophobic interactions can be applied to many kinds of drugs [15] because most drugs contain hydrophobic moiety(ies) in their chemical structures. The second characteristics is size of polymeric micelles, which is in a diameter range approximately from 20 to 60 nm. This range of diameter is smaller than obtainable diameters of liposomes and micro(nano)spheres with considerable stability. The smaller carrier systems are expected to show the higher vascular permeability at target sites by diffusion mechanism. Furthermore, the diameter range of the polymeric micelle systems is considered to be appropriate to evade renal excretion and non-specific capture by the reticuloendothelial systems.

In our previous papers having reported high in vivo antitumor activities [6,12], a ratio between the chemically conjugated and physically entrapped ADR was not determined [16], and considerable amounts of adriamycin derivatives formed and were incorporated in the micelles. Our recent paper reported an improved ADR incorporation method which can determine this ratio and reduce amounts of adriamycin derivatives as impurities [17]. In this paper, the ADR incorporated in the inner core was quantitatively measured using the improved synthetic method, and effects of the ADR contents (both by chemical conjugation and physical entrapment) on micelle stability and in vivo antitumor activity were analyzed. By these analyses, a strategy in designing polymeric micelles for anticancer drug delivery was obtained.

2. Experimental part

2.1. Materials

Adriamycin hydrochloride (ADR·HCl) was purchased from Mercian Corp., Japan. Other chemicals were of reagent grade and were used as purchased.

2.2. Chemical conjugation and physical entrapment of adriamycin to block copolymer

Procedures for chemical conjugation and physical entrapment of adriamycin (ADR) into polymeric micelles were based on a reference [17] with some modifications.

2.2.1. Chemical conjugation of ADR to block copolymer

Synthesis of poly(ethylene glycol)-poly(aspartic acid) block copolymer [PEG-P(Asp)] was reported in a reference [16]. Molecular weight of the poly(ethylene glycol) (PEG) chain and the poly(aspartic acid) (P(Asp)) chain was 12 000 and 2100, respectively. The content of the β-amide form of the aspartic acid residues was found ca. 75 mol.% (x:y=1:3 in Fig. 1). PEG-P(Asp) was dissolved in N,N-dimethylformamide (DMF), and adriamycin hydrochloride (ADR·HCl) and triethylamine (1.3 mol. equivalents to ADR) were consecutively added to the block copolymer solution. The mixture was cooled to 0°C, and the conjugation reaction was initiated by an addition of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC·HCl). Four hours following the reaction at 0°C with stirring, EDC·HCl was again added. Twenty hours after the second addition of EDC·HCl, the reaction mixture was dialyzed with a Spectrapor 2 dialysis membrane, followed by ultrafiltration using an Amicon ultrafiltration membrane PM 30 (MWCO=30 000) in distilled water. Details of the dialysis conditions are summarized in Table 1. Content of the chemically conjugated ADR in PEG-P(Asp(ADR)) with respect to the aspartic acid residues was determined by measuring an amount of unreacted ADR in the reaction mixture by reversed-phase chromatography. By subtracting this amount from that of the added ADR as a substrate, an amount of the conjugated ADR was obtained.

2.2.2. Physical entrapment of ADR into PEG-P(Asp(ADR))

The ADR-conjugated block copolymer [(PEG-P(Asp(ADR))] solution obtained above was dialyzed against DMF using a Spectrapor 2 dialysis membrane for 3 h. A
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Table 1
Chemical conjugati

Run	ADR·HCl (mg)
A	700.7
B	311.2

[a]With respect to A
[b]Dialysis was agai
a→(b)→c.
[c]Out of the total A

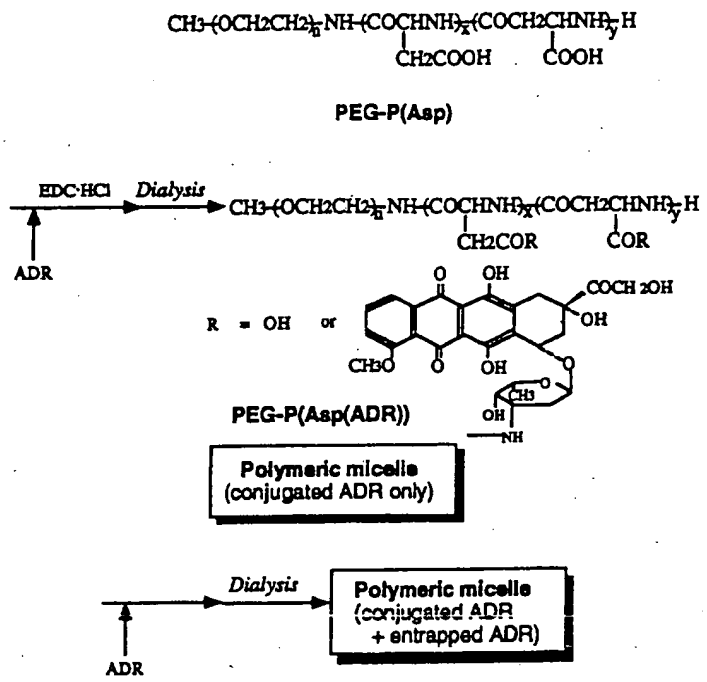

Fig. 1. Preparation of polymeric micelle.

brane for 3 h. ADR·HCl was dissolved in DMF and added by 3.0 mol. equivalents of triethylamine. In run 3, distilled water was further added. The PEG-P(Asp(ADR)) solution in DMF was added to the ADR solution, and this mixture was stirred at room temperature. Then, the mixture was dialyzed against distilled water using a Spectrapor 2 dialysis membrane. Details of the entrapment conditions are summarized in Table 2. An amount of the physically entrapped ADR was determined by reversed-phase chromatography, and the total amount of ADR (physically entrapped ADR+chemically conjugated ADR) was obtained by measuring absorption at 485 nm in distilled water.

2.3. General procedures

HPLC measurements were done at a flow-rate of 1.0 ml/min at 40°C. Detection was performed by absorption at 485 nm.

Reversed-phase chromatography was carried out with a Waters μBondasphere 5μ C4-100Å column (3.9 mm×15 cm, Nihon Waters, Tokyo, Japan) with 20 μl of samples at a concentration of 200 μg

Table 1
Chemical conjugation of ADR to PEG-P(Asp) block copolymer

Run	ADR·HCl (mg)	PEG-P(Asp) (mg)	ADR Asp unit (molar ratio)	DMC (ml)	EDC·HCl (mg)	Ratio of conjugated ADR[a]	Dialysis (h)[b] a	b	c	Residual free ADR in micelle (%)
A	700.7	1130	0.80	77	461+463	0.63	22	–	2	0.6
B	311.2	890	0.45	61	454+453	0.41	2	1	1	0.0

[a]With respect to Asp units.
[b]Dialysis was against; a: 3 vol.% acetic acid in methanol, b: 1 vol.% acetic acid in distilled water, c: distilled water in the order of a→(b)→c.
[c]Out of the total ADR.

Table 2
Physical entrapment of ADR in the polymeric micelles

Run	ADR·HCl (mg)	PEG-P(Asp(ADR)) (ADR equivalent mg)	DMF (ml)	Water (ml)	Stirring time (min)	Dialysis (h)	Proportion of physically entrapped ADR[a] (%)
1	96.1	run A 90.1	40	0	90	3	17.8
2	60.1	run B 60.1	30	0	90	3	8.4
3	60.1	run B 60.1	30	5	10	4	10.2

[a] Out of the total ADR. Physically entrapped ADR was measured by HPLC, and total ADR was measured by absorption at 485 nm.

equivalent ADR/ml (measured by absorption at 485 nm) in a gradient eluent using 1% acetic acid aqueous solution and acetonitrile. Gradient conditions are shown in Fig. 2a. HPLC conditions for identification of the third peak of Fig. 2c are described in a later section.

Gel-permeation chromatography (GPC) was carried out with an Asahipak GS-520 H column in 0.1 M phosphate buffered solution (pH 7.4) with 100 μl of samples at a concentration of 20 μg equivalent ADR/ml (measured by absorption at 485 nm).

2.4. Identification of the third peak of Fig. 2c

2.4.1. Synthesis of a compound of the third peak

ADR·HCl (10 mg) was dissolved in a mixture of 1 ml of DMF and 3 ml of water, followed by an addition of 5 μl of triethylamine. This solution was stirred at room temperature for 5 h.

2.4.2. Model reactions between ADR analogues

Reactions were made between ADR, N-acetyl ADR, daunomycin (DAU), and N-Acetyl DAU. Examined combinations are summarized in Table 4. One milliliter solution of substrate 1 in DMF was mixed with substrate 2 solution in DMF, followed by additions of 5 mg of triethylamine and 1 ml of water. This mixture was incubated at room temperature for 5 h. Concentrations of ADR and DAU solutions were 2 mg/ml, and N-acetyl ADR and N-Acetyl DAU were used immediately after acetylation reactions. N-acetyl ADR and N-acetyl DAU were obtained as

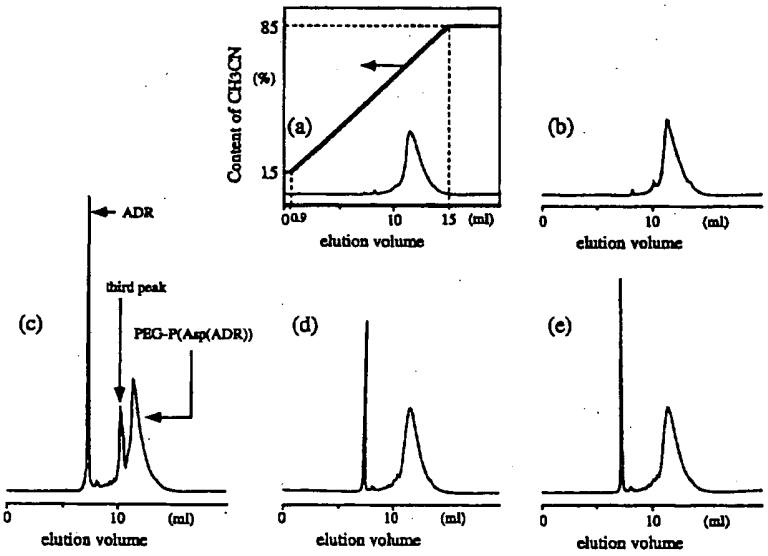

Fig. 2. reversed-phase chromatograms of polymeric micelles listed in Table 1 and 2. (a) run A, (b) run B, (c) run 1, (d) run 2, and (e) run 3. Column; Waters μBondasphere 5 μ C_4-100, sample; 20 μl at a concentration of 200 μg equivalent ADR/ml, detection; absorption at 485 nm, elution; a gradient using 1% acetic acid aqueous solution and acetonitrile. Gradient conditions are shown in Fig. 2(a).

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2.5. In vivo a

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follows. Acetic acid (3 ml) was dissolved in 5 ml of DMF and added by 10 mg of EDC·HCl, followed by incubation at room temperature for 30 min. ADR·HCl or DAU·HCl (10 mg) was dissolved in 5 ml of DMF and added by 2 mg of triethylamine. This solution was mixed with the acetic acid/EDC·HCl solution, and this mixture was stirred at 35°C for 24 h. Complete conversion of ADR/DAU to N-acetyl ADR/DAU was confirmed by HPLC.

2.4.3. NMR measurements of a compound after reduction and acid-hydrolysis

The compound of the third peak was obtained as described in Section 2.4.1, and this reaction mixture was added by 2 mg of $NaBH_3CN$ and stirred at room temperature for 4 h. Then, the reaction mixture was added by 100 μl of HCl (11.8 M), and stirred at room temperature for 24 h. Purification of a product which showed absorbance at 485 nm was carried out by HPLC twice, in the conditions described below (Section 2.4.4) in the first run and in the conditions described above (general procedure) in the second run. The product was applied to SepPakC18. Then, the product was eluted with methanol. Methanol was evaporated, and the residual product was dissolved in DMSO-d_6 containing a small amount of DCl. Measurements were carried out using a JNM-α400 spectrometer (JEOL, Tokyo, Japan).

2.4.4. HPLC analysis

Conditions of HPLC analysis were as follows; column: Waters μ-Bondasphere C_4 (5 μ, 300 Å), flow: 0.8 ml/min, elution: gradient of (a) 0.05% IPCC-MS7 (an ionpair reagent, GL Sciences, Tokyo, Japan) +0.01% trifluoroacetic acid and (b) CH_3CN, and gradient is shown in Fig. 5a.

2.4.5. Mass spectroscopy

ESI-mass spectroscopic measurements were done using a Quattro II mass spectrometer (Micromass Ltd.).

2.5. In vivo antitumor activity

Antitumor activity against a solid tumor of polymeric micelles run A, 1, 2, and 3 and free ADR was evaluated with mouse colon adenocarcinoma 26 (C 26). C 26 cells (1×10^5 cells in 0.2 ml of 0.9% NaCl solution) were transplanted into CDF1 female mice (6 weeks old) subcutaneously on day 0, and drug injection started on day 7 when tumor volume reached approximately 100 mm^3. Drug was injected into a tail vein with a schedule of q4d×3 (on day 7, 11, and 15) in volume of 0.1 ml/10 g body weight. Six mice were included in one group except 12 mice for the control. The mortality was monitored daily, and tumor volume was measured at a few days interval. Tumor volume was calculated as follows: volume=$1/2LW^2$; L is long diameter and W is short diameter. Statistical analysis was done on day 10 after the first drug injection using Dunnett's method.

2.6. In vitro cytotoxicity

In vitro cytotoxic activity of polymeric micelles run A, 1, 2, and 3 and free ADR were assayed against P $388D_1$ mouse leukemia cells. P $388D_1$ cells were incubated with serially diluted drugs (two-fold steps) in 100 μl of RPMI 1640 medium (containing 10% of fetal calf serum and 50 unit/l of penicillin and 50 μg/l of streptomycin) with a Multiwell™ tissue culture plate (96-well) of Falcon 3047 for 24 h or 72 h in 5% CO_2 at 37°C. After the incubation for the defined period, cell viability was determined by MTT assay [18] to obtain the IC_{50} value.

3. Results

3.1. Chemical conjugation and physical entrapment of ADR

As summarized in Table 1, two adriamycin-block copolymer conjugates [PEG-P(Asp(ADR))] were synthesized of 63 mol.% and 41 mol.% substitution of aspartic acid (Asp) residues of the block copolymer with adriamycin (ADR) for run A and run B, respectively. Immediately after the conjugation reaction, 17 mol.% and 4 mol.% of ADR with respect to the Asp residues remained unconjugated for run A and run B, respectively. These unconjugated ADR were efficiently removed by dialysis against methanol containing 3% acetic acid (and 1% acetic acid aqueous solution for run B). As a result, less than 1% of the physically entrapped ADR out of

the total ADR (physically entrapped+chemically conjugated) remained in the polymeric micelles, as shown in reversed-phase chromatograms of Fig. 2 a and b; no or a very small peak of unconjugated ADR was seen at 7.3 min, while the conjugated ADR (PEG-P(Asp(ADR))) eluted as a broad peak with a peak top around 11.5 min.

ADR was successfully entrapped by physical manner into polymeric micelles forming from PEG-P(Asp(ADR)) as summarized in Table 2. In run 1 and 2, ADR was mixed with PEG-P(Asp(ADR)) in almost the same amount as that of the chemically conjugated ADR of PEG-P(Asp(ADR)), considerable amounts of ADR were physically entrapped in the micelles. This entrapment efficiency was dependent on the content of the chemically conjugated ADR in PEG-P(Asp(ADR)). In comparison between run 1 and run 2, more than double content of the physically entrapped ADR was obtained by using PEG-P(Asp(ADR)) run A, which had the higher content of the chemically conjugated ADR. This shows that non-covalent interactions between the chemically conjugated ADR and the added ADR preferentially occurred in the more concentrated atmosphere of the chemically conjugated ADR. Slightly higher physical entrapment (10.4%) was obtained in run 3 by mixing the substrates in a mixture of DMF and distilled water for a shorter period. For run 1, a new peak appeared at 10.4 min in a reversed-phase chromatogram. Characterization of this third peak at 10.4 min is described in a later part.

All five samples (run A, B, and 1–3) were observed to form micellar structures by polymeric micelle peaks eluting near the gel-exclusion of the column. Elution volumes from 4.4 to 5.3 ml correspond to much larger molecular weights than those of the ADR-block copolymer conjugates (M.W.: run A; 20 200, run B; 18 000). However, precise peak positions differed among them. Delay in elution is considered to result from some interactions of the polymeric micelles with gel of the column. In comparison between Fig. 3a and b, a peak at the larger elution volume was seen for PEG-P(Asp(ADR)) with the lower content of the chemical conjugated ADR. This indicates that the polymeric micelle forming from PEG-P(Asp(ADR)) run B interacted with gel in the column more strongly than the micelle from run A probably due to looser packing of the hydrophobic inner core of run B.

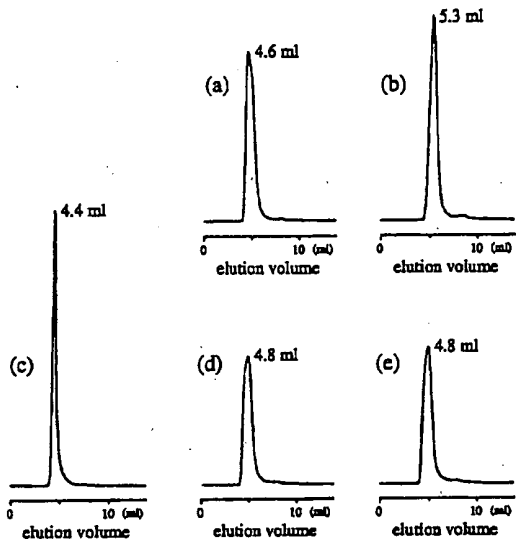

Fig. 3. Gel-filtration chromatograms of polymeric micelles. (a) run A, (b) run B, (c) run 1, (d) run 2, and (e) run 3. Column; Asahipak GS-520 H column, eluent; 0.1 M phosphate buffered solution (pH 7.4), sample; 100 μl at a concentration of 20 μg equivalent ADR/ml.

Thus, the content of the chemically conjugated ADR was found to influence stability of the micellar structures, and the stability of the micellar structure could be relatively evaluated by this gel-exclusion chromatography. By physical entrapment of ADR into the polymeric micelles, micelle peaks were observed to shift to smaller elution volumes for both PEG-P(Asp(ADR)) run A and B. Elution volume shifted from 4.6 ml (Fig. 3a) to 4.4 ml (Fig. 3c) for run A, and from 5.3 ml (Fig. 3b) to 4.8 ml (Fig. 3d and e) for run B. These results show that stability of the micellar structure increased by additions of the physically entrapped ADR probably due to increased hydrophobic interactions inside the inner cores of the micelles. These all results indicate that both the contents of the chemically conjugated ADR and physically entrapped ADR influenced the micelle stability measured by gel-filtration chromatography as well as chain lengths of the two segments of the block copolymer did in our preceding paper [19].

3.2. Identification of chemical structure of the third peak of Fig. 2c

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2) ADR and

Table 3
Number of product

Substrate 2
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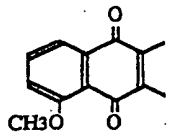
N-Ac ADR

1) N-AcADR: N-a



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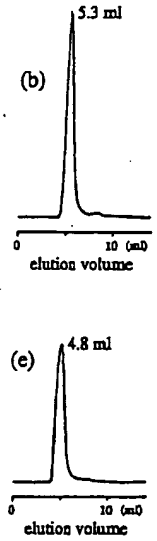

meric micelles. (a) run
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m of run 1, the

third peak other than intact ADR (7.3 ml) and the chemically conjugated ADR (11.5 ml) was observed at 10.4 ml in elution volume. In order to identify a chemical structure of this third peak, conditions of the reversed-phase chromatography were optimized to separate the third peak from the chemically conjugated ADR. When 0.05% IPCC-MS7 (an ion-pair reagent, GL Sciences, Tokyo, Japan) +0.01% trifluoroacetic acid in distilled water was used as one component of gradient eluent on behalf of 1% acetic acid, the third peak was separated from the other two peaks, as shown in Fig. 5a. This third peak was collected and analyzed by ESI-mass-spectroscopy. Molecular weight of this peak was revealed to be 1067.3. This value implies that this third peak was a dimer (derivative) of ADR molecules. This compound was also obtained by mixing ADR·HCl and triethylamine in DMF. The chemical structure of this peak was identified as shown in Fig. 4 by the following experimental results.

1) When this compound was incubated in a 30:70 (vol.) mixture of 1% acetic acid aqueous solution and CH_3CN at room temperature for 3 days, this compound was observed to change into equivalent amounts of ADR and the other compound in a reversed-phase chromatogram detected by adsorption at 485 nm. This result indicates that an acid-labile linkage was included in this compound.

2) ADR and 3 adriamycin analogues were mixed

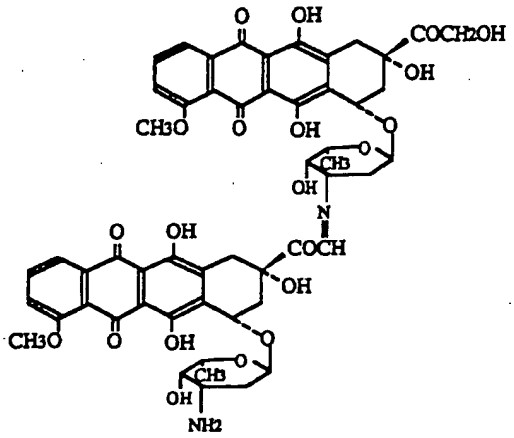


Fig. 4. Chemical structure of the third peak of Fig. 2(c).

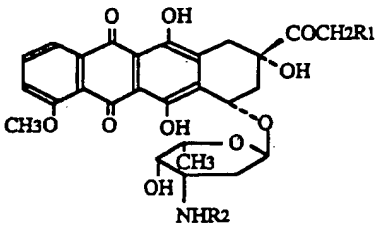
in DMF with an addition of triethylamine, the number of products was dependent on combinations as summarized in Table 3. These numbers can be understood by the hypothesis that the products were obtained by reactions between a primary amino group (of ADR or daunomycin (DAU)) and a reactive carbonyl group of ADR or N-acetyl adriamycin.

3) This compound was treated with $NaBH_3CN$, followed by treatment with HCl. NMR spectrum of the product was measured in DMSO-d_6 containing a small amount of DCl. As shown in Fig. 5b–d, this

Table 3
Number of products from adramycin and its derivatives

Substrate 2	Substrate 1			
	ADR	N-Ac ADR	N-Ac DAU	DAU
ADR	1	2	1	2
N-Ac ADR	2	0	0	1

1) N-AcADR: N-acetyl adriamycin, DAU: daunomycin, and N-Ac DAU: N-acetyl daunomycin.



	R_1	R_2
ADR	OH	H
N-Ac ADR	OH	$COCH_3$
DAU	H	H
N-Ac DAU	H	$COCH_3$

2) Reaction conditions; One milliliter solution of substrate 1 (2 mg/ml) in DMF was mixed with substrate 2 solution (2 mg/ml) in DMF, followed by additions of 5 mg of trietylamine and 1 ml of water. This reaction mixture was incubated at room temperature for 5 h.

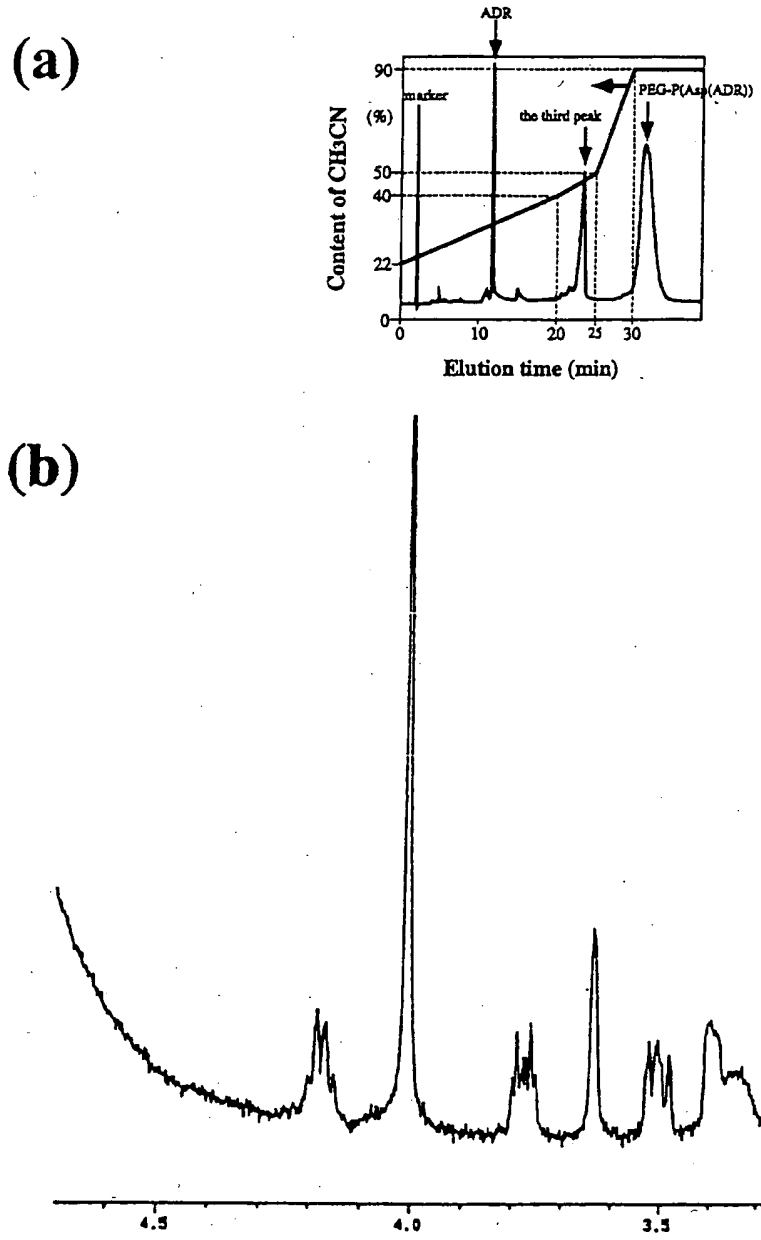
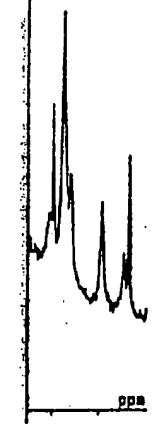


Fig. 5. Identification of the third peak of Fig. 2(c). (a) reversed-phase chromatogram; column: Waters μ-Bondasphere C_4 (5 μ, 300 Å), flow: 0.8 ml/min, elution: gradient of 0.05% IPCC-MS7 (an ionpair reagent)+0.01% trifuoroacetic acid and CH_3CN, gradient profile is shown in the figure. (b) ^{1}H-NMR spectrum, (c) COSY spectrum, and (d) CH COSY spectrum of the third peak after treatments with $NaBH_3CN$ and HCl.



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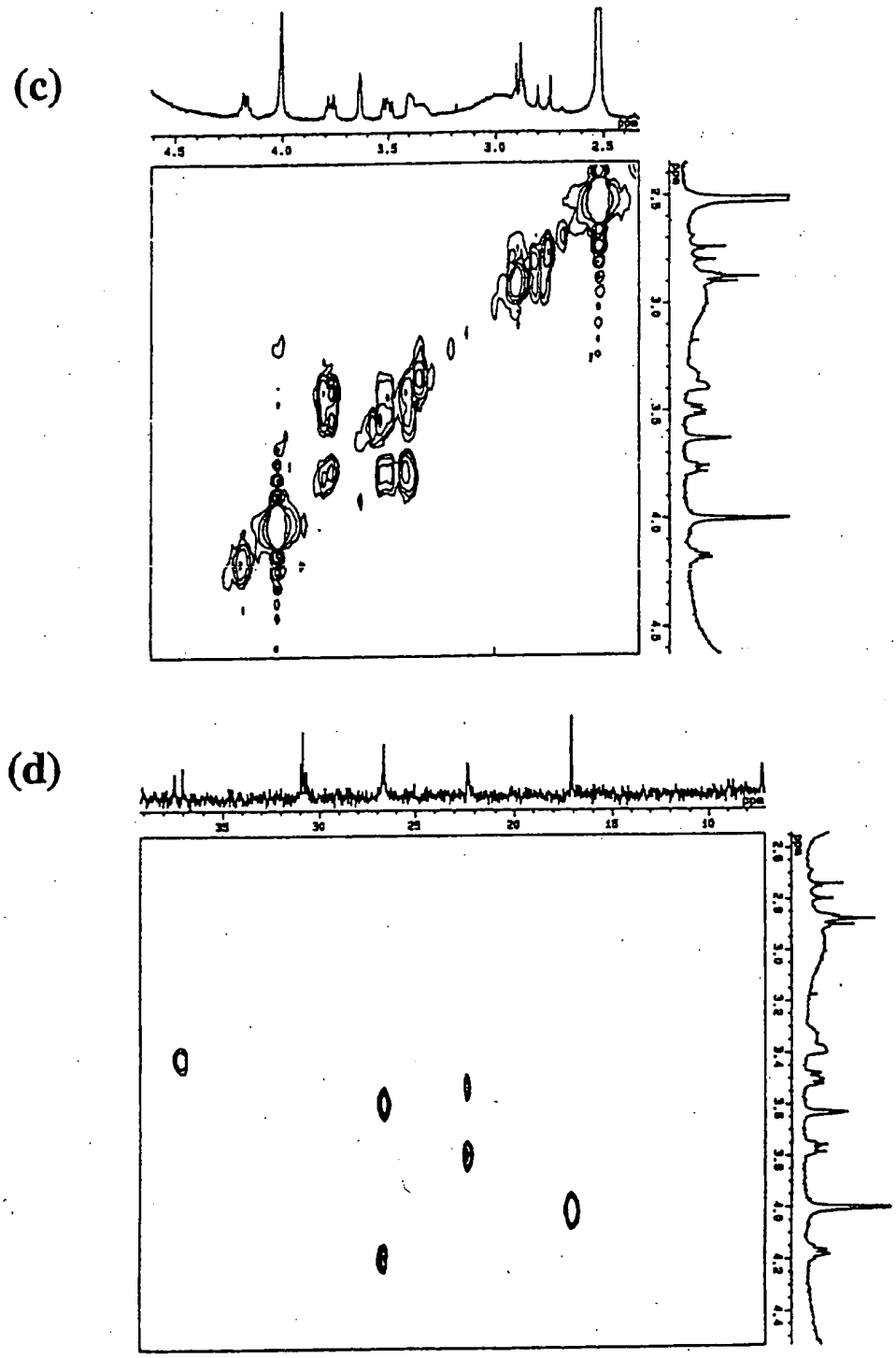

Fig. 5. (*continued*)

product contained two protons at 3.50 ppm and 3.77 ppm, which were coupled with each other in COSY spectrum (Fig. 5c) and coupled with carbon at 61.8 ppm that was assigned as a methylene next to a carbonyl and a secondary amine in CH COSY spectrum (Fig. 5d). This product also contained a carbonyl carbon at 190.3 ppm, that is other than a carbonyl carbon of adriamycin molecule. From these results, it was elucidated that this compound contained -$COCH_2NH$- after treatments with $NaBH_3CN$ and HCl.

3.3. In vivo antitumor activity

Fig. 6 shows in vivo antitumor activities against C 26 tumor. Relative tumor volume from the day of the first intravenous injection is logarithmically plotted. In Fig. 6a, intact adriamycin showed significant inhibition effect on tumor growth only at the maximum tolerated dose (10 mg/kg, $p<0.15$ on day 10). A higher dose (20 mg/kg) resulted in toxic deaths, and a lower dose (5 mg/kg) did not show significant tumor growth inhibition. One composition of the polymeric micelle (run 1) showed significant antitumor effect at a wider dose range than that of intact ADR, as shown in Fig. 6c. The polymeric micelle run 1 showed significant reduction of tumor volume at 2 doses; 50 mg total ADR/kg per day $p<0.05$ on day 10 after the first injection, 25 mg total ADR/kg per day $p<0.1$ on day 10 (total ADR means the chemically conjugated plus physically entrapped ADR). At this maximum tolerated dose (50 mg/kg), tumor completely disappeared in 2 mice out of 6. Such obtained high activity was at the same level of the polymeric micelle that was prepared by the old preparation method [6]. In Fig. 4b, no significant antitumor effect was observed for run A which lacked the physically entrapped ADR but had the same amount of the chemically conjugated ADR as that of run 1. This comparison indicates that the physically entrapped ADR was required to express significant antitumor effects against C 26 tumor. The other two compositions of micelles (run 2 and 3) with the lower amount of the chemically conjugated ADR did not express any significant antitumor activity, even though run 2 and run 3 at a dose of 50 mg/kg contained almost the same amount of the physically entrapped ADR as that of run 1 at a dose of 25 mg/kg. These results indicate that antitumor activity was dependent on the ADR contents of the polymeric micelles.

3.4. In vitro antitumor activity

Cytotoxic activity of the polymeric micelles against P388D1 mouse leukemia cells are summarized in Table 4, where IC_{50} values are shown by the total (chemically conjugated+physically entrapped) ADR. All the polymeric micelle samples expressed incubation time-dependent cytotoxicity, and their IC_{50} values were much larger those of intact ADR both for 24 and 72 h incubation. When these values are multiplied with ratios of the physically entrapped ADR in the total ADR, the multiplied values are almost the same as with those of intact ADR: values between 0.16 and 0.19 are obtained in the three micelle samples for 24 h except run A (IC_{50} of intact ADR was 0.17) and values from 0.0027 to 0.0034 are obtained in all the micelle samples for 72 h incubation (IC_{50} of intact ADR was 0.0039). These facts suggested that only the physically entrapped ADR expressed cytotoxic activity, while the chemically conjugated ADR did not for the incubation periods up to 72 h. However, the chemically conjugated ADR might express cytotoxic activity in vivo where the polymeric micelle could reside at tumor sites for a longer period than 72 h.

4. Discussion

Large enhancement of antitumor activity of ADR was successfully achieved by its incorporation into polymeric micelles with the controlled amounts of the chemically conjugated and physically entrapped ADR. The high in vivo antitumor activity of run 1 is considered to result from selective delivery of the physically entrapped ADR to solid tumor sites, since the block copolymer-drug conjugate run A did not show any in vivo activity. Furthermore, in vivo antitumor activity was found to depend on contents of the physically entrapped and chemically conjugated ADR; run 1 showed the activity, and run 2 and 3 did not. Gel-filtration analyses revealed differences in micelle structural stability. The polymeric micelle with high structural stability shown by a small

Fig. 6. In vivo a
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(a) □: Control,
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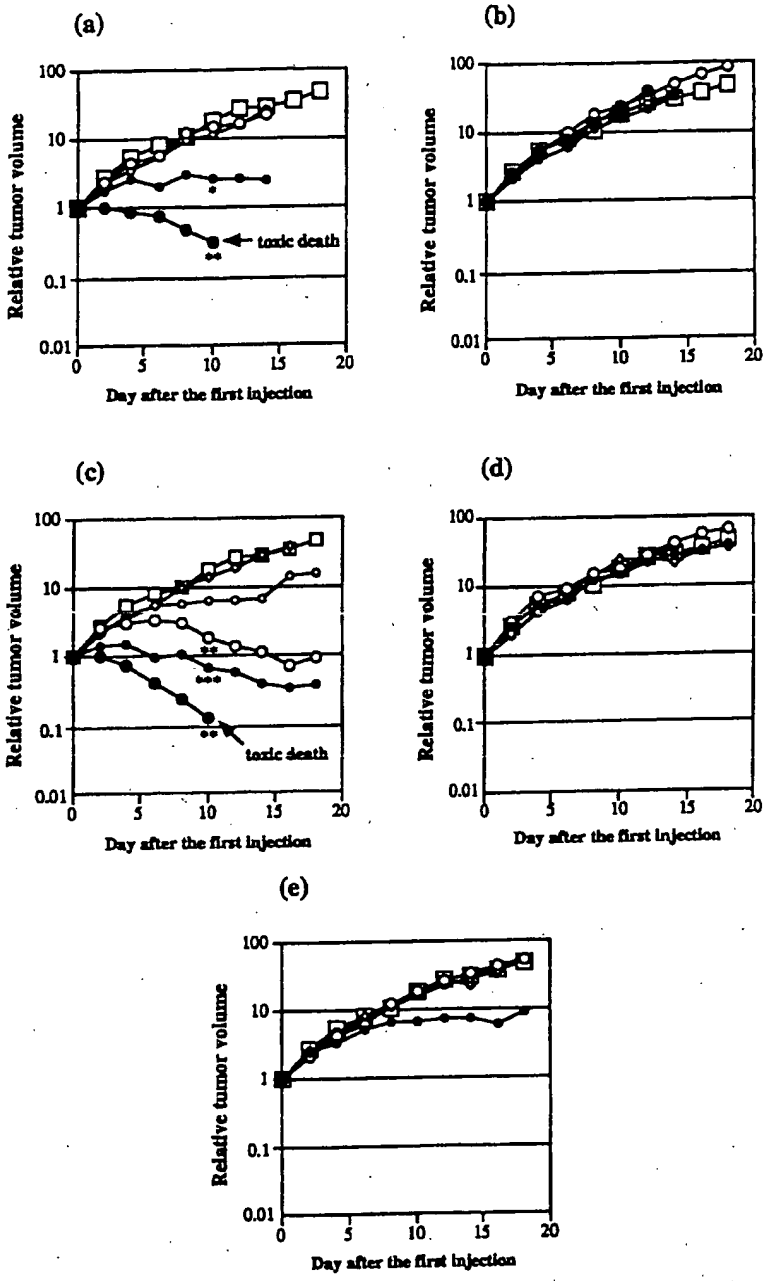

Fig. 6. In vivo antitmor activity of ADR and polymeric micelles against C 26. (a) ADR, (b) run A, (c) run 1, (d) run 2, and (e) run 3. CDF_1 female mice (6 weeks old) were subcutaneously transplanted with C 26 cells. Eight days after the tumor transplantation, drug injection started. Drug was injected intravenously three times on day 0, 4, and 8. Data with four or less surviving mice are not plotted. Plot symbols; (a) □: Control, ●: ADR 20 mg/kg, ●: ADR 10 mg/kg, ○: ADR 5 mg/kg, ◦: ADR 2.5 mg/kg, (b)–(e) □: Control, ●: total ADR 100 mg/kg, ●: total ADR 50 mg/kg, ○: total ADR 25 mg/kg, ◦: total ADR 12.5 mg/kg, and ◇: total ADR 6.3 mg/kg. Statistical analysis was done by a Dunnett's method on day 10; *** $p<0.05$, ** $p<0.1$, and * $p<0.15$.

Table 4
In vitro cytotoxicity of polymeric micelles against P388D1.

	IC_{50} (ADR equivalents μg/ml)[a]	
sample	24 h incubation[b]	72 h incubation[b]
run A	n.d.[c]	0.57
run 1	0.92	0.017
run 2	2.3	0.032
run 3	1.6	0.033
ADR	0.17	0.0039

[a] IC_{50} values are shown by the total (chemical conjugated and physical entrapped) ADR.
[b] Initial cell concentration: 24 h incubation; 7.8×10^5 cells/ml, 72 h incubation 4.3×10^3 cells/ml.
[c] n.d.; not detected up to 118 μg/ml.

elution volume expressed high in vivo antitumor activity. These results indicate that stable physical entrapment of ADR was essential for in vivo antitumor activity in this polymeric micelle drug carrier system.

In our preceding report [9], polymeric micelles were found to selectively accumulate at C26 tumors by radioisotope label on block copolymers. This selectivity was considered to be based on hyperpermeability of the vascular endothelia at tumor sites [20,21]. This unique phenomenon was clearly defined as enhanced permeability and retention effect (EPR effect) for a drug targeting strategy to solid tumors [22,23]. Although this EPR effect was originally reported for proteins such as albumin, it may be applied also to polymeric micelles that have much larger diameters than those of the proteins. In order to utilize the EPR effect for drug targeting to solid tumors, interactions (e.g. hydrophobic interactions) of drug carrier systems with the vascular endothelial cells should be avoided, since these hydrophobic interactions may considerably reduce contribution of diffusive and convectional transport (through intracellular channels or intercellular junctions of endothelia) on which the EPR effect is based. The polymeric micelles with the PEG outer shells are considered to inherently possess selective targeting ability to tumors by the EPR effect, since PEG is known to be an inert polymer with very small interactions with cells and proteins, as demonstrated by prolongation of plasma half-lives of several proteins by PEG modification [24,25]. Stability of the polymeric micelles measured by gel-filtration chromatography is considered to show how completely the hydrophobic inner cores were shielded by the PEG outer shells. Consequently, the stable polymeric micelles could more effectively utilize the EPR effect by more efficiently inhibiting the hydrophobic interactions. To obtain the stable entrapment, large amounts of the chemically conjugated and physically entrapped of ADR were required probably for tight packing of the hydrophobic inner cores.

It is considered that the chemically conjugated ADR did not play a major role in antitumor activity, since polymeric micelle run A did not show any in vivo activity and did show no or low in vitro cytotoxic activity. Such no or low activities are considered to result from no or slow (if any) release of free ADR from the block copolymer due to the absence of cleavable spacer groups such as tetrapeptide of Kopecek's study [2]. However, it still remains unknown whether the chemically conjugated ADR could express any pharmacological effects or not. According to Tritton's study [26], the conjugated ADR can express cytotoxic activities without any release of free ADR. Further studies will be done to clarify this point. On the other hand, the chemically conjugated ADR was shown to contribute to physically entrap free (unconjugated) ADR in a stable manner inside the inner core, since no physical entrapment of ADR was attained by mixing ADR and poly(ethylene glycol)-poly(aspartic acid) block copolymer (data not shown). Martin et al. [27] reported self-association of daunomycin (an adriamycin analogue). Therefore, the conjugated ADR is considered to work as a very good carrier of the physically entrapped ADR by providing specific interactions (e.g. π-π interaction) between the two ADR molecules as well as non-specific hydrophobic interactions.

A dimer was found to form during the physical entrapment of ADR for run 1. The content of this dimer (8.6% out of the total ADR, obtained from its peak area in Fig. 2c in the polymeric micelle run 1 was smaller than that of ADR (17.8%). There are several possibilities for roles of this dimer in antitumor activity in vivo. The first one is that the dimer does not possess any particular significance on antitumor activity or selective delivery. In other words, antitumor activity and selective delivery only depend on a total quantity of the physically entrapped ADR i
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trapped ADR and this dimer. This hypothesis, however, is unlikely because of significant difference in hydrophobicity between the dimer and ADR. On the other hand, this dimer may be important for the in vivo activity due to its more hydrophobic character than ADR, which was shown by its larger elution volume in reversed-phase chromatography than ADR. The dimer can work in the following two possible ways; (1) the dimer played a more important role in cytotoxic activity at tumor sites than ADR by more efficient delivery to tumor sites due to its smaller release from the micelles in the bloodstream, (2) The dimer contributed to stably entrap ADR, which played a major role in cytotoxic activity at tumor sites. Further study is currently under way to elucidate these points.

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